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14. ABSTRACT

During the performance period, we screened a phage display library of ~5x109 individual 12-mer peptides for specific binding to a pentaglycine substrate. The pentaglycine substrate is a synthetic mimetic of the cross bridge found in the cell walls of Staphylococcus aureus. We purified, sequenced, and identified 40 candidate peptides that bound the pentaglycine target higher than bovine serum albumin control screens as determined by enzyme-linked immunosorbant assays (ELISA). Of these 40 candidates, we further screened the phage displaying the top 10 tightest binding peptides against S. aureus in a spun-ELISA assay. We found two peptides, #5 and #39, bound to whole staphylococcal cells as well as pentaglycine. Next, we added the phage displaying #39 or a randomized version of #39 to growing staphylococci and showed that #39, but not the randomized version, significantly slowed growth of the bacteria. We next chemically synthesized all 10 of our candidate peptides, randomized versions of all 10, and fluorescent-labeled versions of all 10. Unfortunately, none of the peptides alone displayed an inherent antimicrobial capacity but some showed synergy with oxacillin against methicillin-resistant staphylococci as well as synergy with vancomycin against vancomycin-resistant staphylococci. We then used fluorescent derivatives of the peptides to show binding to the staphylococcal surface. Finally, we conjugated one peptide (#2) to vancomycin through a thiol group and showed it had similar, but not better, properties than unlabeled vancomycin.

15. SUBJECT TERMS

Bacteriophage, Vancomycin, Phage Display, Binding Energy, Antibiotic Resistance, Staphylococcus

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INTRODUCTION:

Modification to the site of antibiotic binding is one of the most widely used mechanisms of antibiotic resistance in bacteria. Even minor losses in affinity result in a significant excess of antibiotic required to achieve the same level of effectiveness. Unfortunately, these levels often exceed toxicity limits, effectively making the organism resistant to clinically safe doses the antibiotic. Thus, "resistance" in these cases is equivocal to "loss of affinity". As an example, vancomycin is a glycopeptide antibiotic that binds to the Ala-Ala cell wall precursor of Gram-positive bacteria through five hydrogen bonds, thereby blocking transpeptidation (linking) of a crossbridge connecting two separate cell wall strands. However, in vancomycin-resistant strains, a terminal lactate residue (Lac) is present in lieu of an alanine residue (Ala) on the peptide stem precursor (i.e., Ala-Lac instead of Ala-Ala). Although vancomycin will bind Ala-Lac with four hydrogen bonds, the conversion of the last alanine residue to a lactate moiety causes a 1000-fold drop in binding affinity, rendering the organism clinically resistant to vancomycin. We propose that an increase in antibiotic affinity for the mutated target-site would "re-sensitize" the organism to the antibiotic for which it had previously developed resistance. To achieve this, we will screen bacteriophage-display libraries for random peptides that bind to the unique pentaglycine cross-bridge of the *Staphylococcus aureus* cell wall. Once identified and validated, the peptide(s) will be conjugated to vancomycin. In this scheme, the peptide will act as "molecular glue" that would supply the missing binding energy needed for the antibiotic to inhibit transpeptidation of the cell wall in vancomycin-resistant *S. aureus*. This proposal aims to recycle readily available antibiotics by "re-engineering" them back into clinically relevant drugs.

BODY:

Aim 1. Perform phage display to identify putative peptides that bind polyglycine.

Task 1. Obtain reagents. (month 1)

All reagents successfully obtained.

Task 2. Perform biopanning, enrichment, and initial screening of phage display libraries. (months 1-6)

The pentaglycine cross bridge of the *S. aureus* cell wall was immobilized on glass and served as the target epitope for bio-panning against a commercial bacteriophage display library (work done in collaboration with Dr. Joe Fralick) of ~$5x10^9$ individual 12-mer peptides for specific binding. After four rounds of bio-panning/enrichment, 40 phage isolates were assessed by enzyme-linked immunosorbant assay (ELISA) for their affinity toward pentaglycine versus bovine serum albumin (BSA), a non-target protein (Fig. 1). Of these, the ten top binders (#2, 5, 9, 10, 28, 29, 37, 38, 39, and 40) were tested for binding directly to *S. aureus* (below in Task 3).

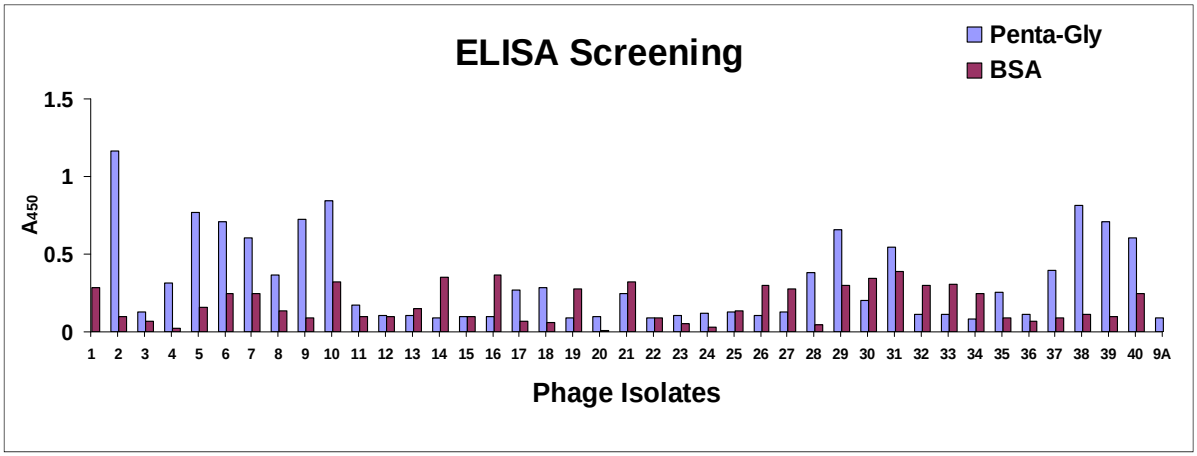

Figure 1. Forty display phage isolates were screened for affinity to pentaglycine and BSA by ELISA. Note, phage 9A is a control phage that does not have any peptide displayed.

Task 3. Test top binding display phage against whole staphylococci. (months 7-9)

Despite being covalently attached to a large filamentous phage, peptides displayed on phages #5 and #39 showed specific binding to live *S. aureus* cells as measured by a spun cell ELISA (Fig. 2). These data suggest the pentaglycine crossbridge must be partially exposed, an assertion which is supported by evidence that anti-staphylococcal antibodies directed against pentaglycine can bind whole bacterial cells. Finally, phage #39 was mixed with growing *S. aureus* cells and was shown to cause a delay in growth (Fig. 3). In contrast, phage #9A, a control phage that does not display any peptide, had no effect on the growth curve.

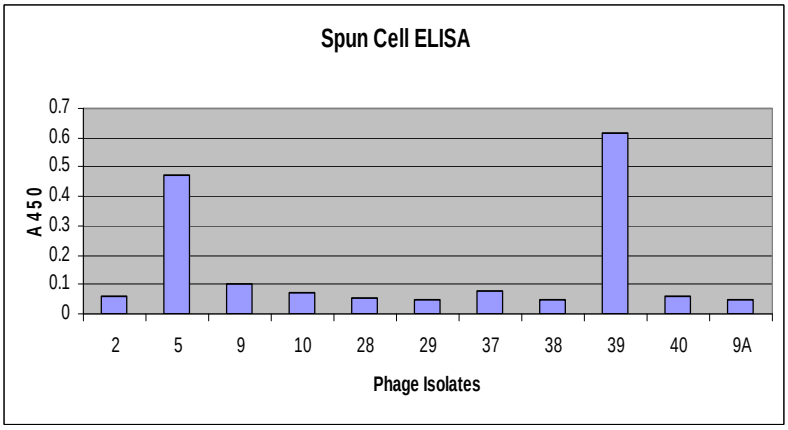

Figure 2. The top ten binding phage from Fig. 1 were screened against whole *S. aureus* by ELISA. Note, phage 9A is a control phage that does not have any peptide displayed.

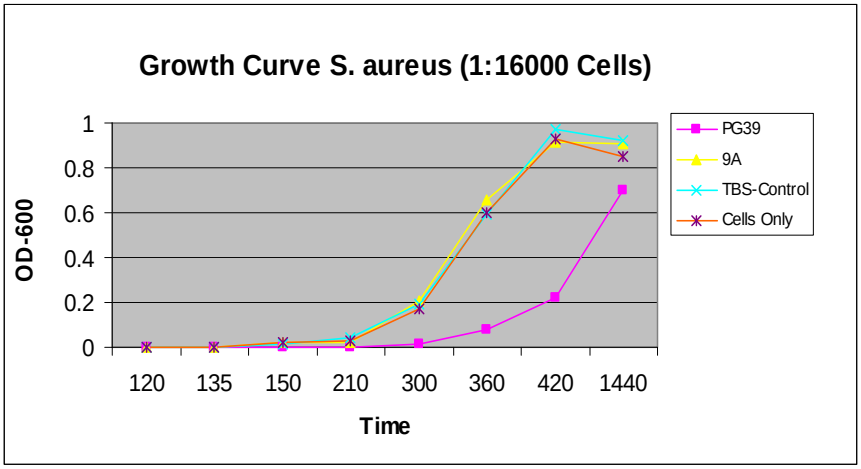

Figure 3. Growth curves of phage #39, control phage 9A, or buffer controls mixed with growing *S. aureus*.

Aim 2. Biochemically characterize pentaglycine/*S. aureus* binding peptides.

Task 1. Chemically synthesize the 10 peptides as well as randomized version of each peptide to serve as controls. (months 9-10)

All the above experiments utilized displayed peptides that were still attached to the bacteriophage. Therefore, we sequenced all 10 phage and determined the identity of each peptide (Figure 4). Significantly, many common motifs emerged from the 10 sequences. Specifically, PK or KP, QS or SQ, AKXP, HXH and HXHXH were seen in peptide after peptide. These common motifs from a random library of ~$5x10^9$ individual clones greatly increased enthusiasm that we found binding epitopes that recognized the pentaglycine crossbridge of the staphylococcal cell wall. As a result, we chose to chemically synthesize all 10 of our top binding peptides, as well as fluorescently labeled derivatives of these peptides, and randomized derivatives of these peptides for further study.

PG2: SPKHDNHTMRLK
PG5: KPAHVMSHNGHR
PG9: KPSPQHYHQHVR
PG10: KPQSHSHPHSQL
PG28: KPTHPHAVHTYH
PG29: AKNPHSHLHYGR
PG37: HMGGHPRHSKLL
PG38: HYNHSPKPLRFK
PG39: AKLPHPHPHSQR
PG40: AKPHTHAIKSTH

Figure 4. Sequence of the top 10 candidate peptides identified by phage display. Common sequence motifs are noted by color coding of the amino acids.

Task 2. Test binding and antimicrobial properties of synthesized peptides. (months 11-13)

We were hopeful that the peptides alone might block the transpeptidation of the cell wall and act as an antibiotic without the need to be conjugated to vancomycin. Unfortunately, we did not observe any killing or inhibition of growth on the *S. aureus* strains we tested. While we have literally done hundreds of assays, a few selected examples of the raw data are shown to demonstrate that none of the peptides had inherent antimicrobial activity. Figure 5 shows eight of the peptides tested on overnight growth of two different staphylococcal strains. While there is some slight variability in the replicate samples, none of the peptides significantly inhibited growth of the pathogen. Likewise, Figure 6 shows a similar trend at different starting concentrations of bacteria or over a concentration range of the peptides.

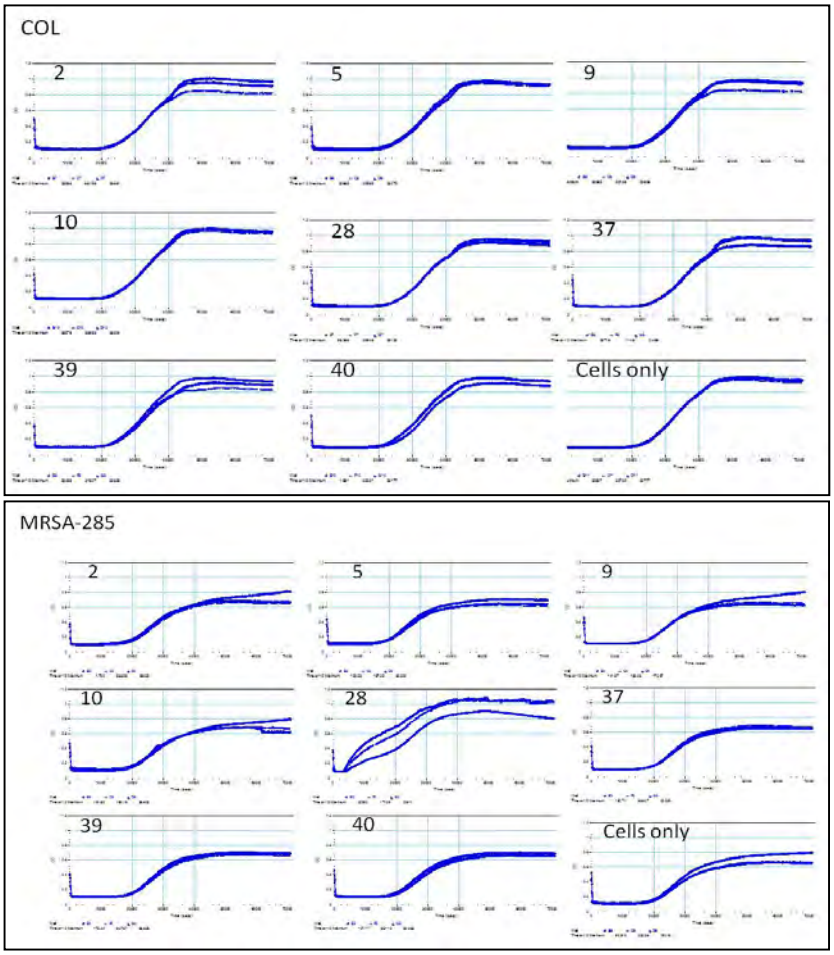

Figure 5. Overnight growth curves of *S. aureus* strains COL and MRSA-285 when exposed to 100 ug/ml of peptides #2, 5, 9, 10, 28, 37, 39, and 40.

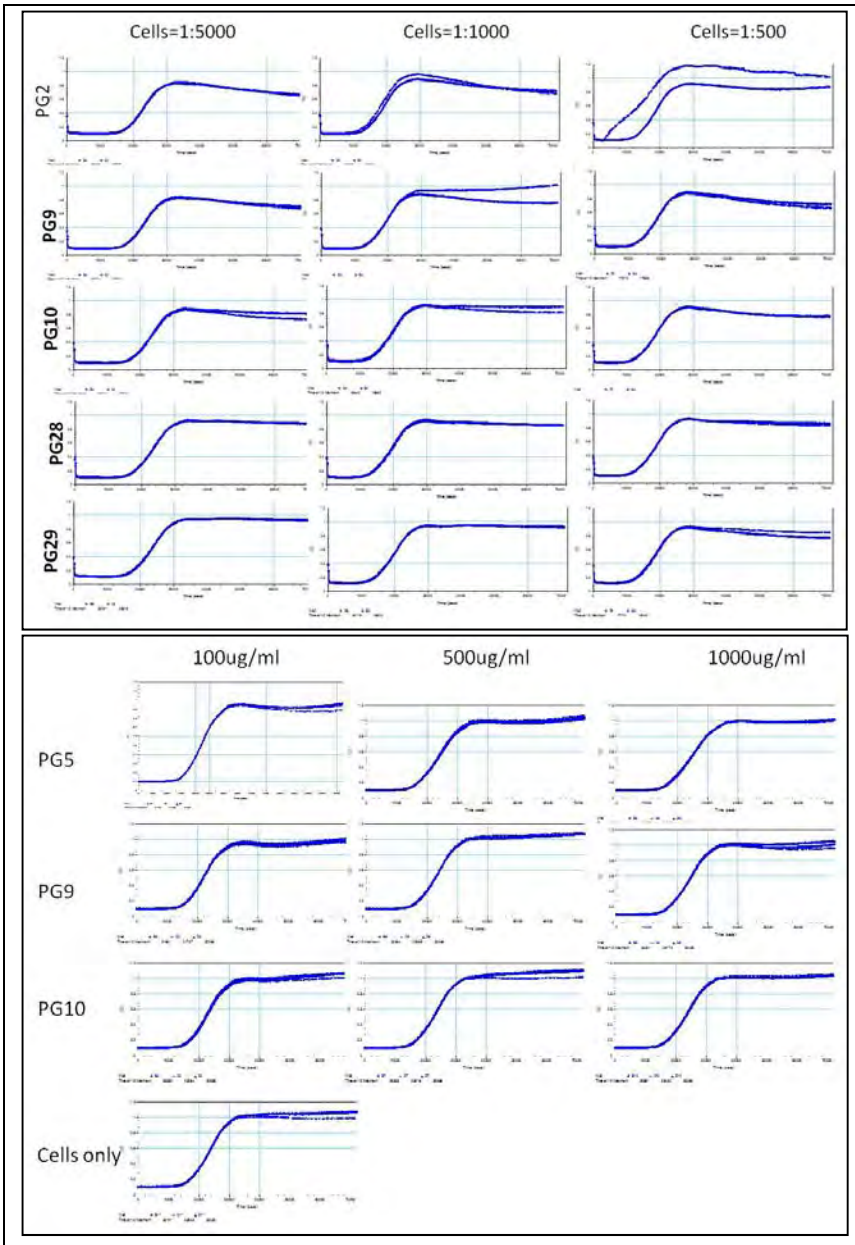

Figure 6. Overnight growth curves of MRSA-285 when exposed to 100 ug/ml of peptides #2, 9, 10, 28, or 29 at different starting concentrations of bacteria (top). Overnight growth curves of MRSA-285 when exposed to 100 ug/ml, 500 ug/ml, or 1000 ug/ml of peptides 5, 9, or 10.

Although none of the peptides inhibited growth of staphylococci, we next wanted to determine if they directly bound to the staphylococcal cell wall. Various biophysical techniques, such as fluorescence polarization anisotropy, isothermal titration calorimetry, and surface plasmon resonance, were tried to measure binding of the peptides to the cell wall with limited success (data not shown). Part of the problem may have been heterogeneity of the cell wall and the methods used to isolate and fractionate the peptidoglycan. Nonetheless, we were able to demonstrate binding by classical microscopy when we used fluorescently labeled derivatives of our peptides. An example is shown in Figure 7.

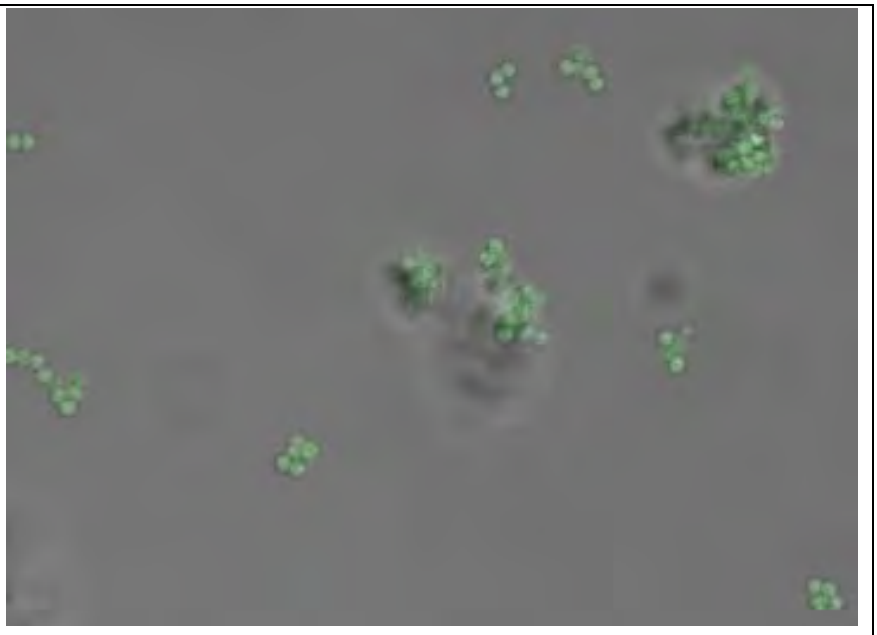

Figure 7. MRSA-285 cells exposed to 10 ug/ml FITC-labeled peptide #5. The image is a merger between bright field and fluorescent images at 1000X magnification.

Because the peptides displayed binding to the staphylococcal cell wall but were not antimicrobial by themselves, we undertook a series of experiments to determine if these peptides displayed synergy with antibiotics. If they can reduce the MIC levels of antibiotics to non-toxic levels, then these peptides may have utility without the need to be conjugated directly to the antibiotics. Toward this end, we undertook several synergy experiments, a few of which are shown below. In Figure 8, a MRSA strain with an MIC>16 ug/ml for oxacillin was evaluated. As can be seen in the figure, 50 ug/ml peptide #2 had no effect on the overnight growth of the bacterial strain. However, when this same amount was mixed with sub-MIC levels of oxacillin (4.6 ug/ml or 2.3 ug/ml), a pronounced bacteriostatic effect was observed. A similar synergy pattern can be seen in Figure 9, where a vancomycin-resistant strain (MIC>256 ug/ml) was tested. Once again, 50 ug/ml of peptide #2 showed a dose-response synergy at sub-MIC levels of vancomycin (50 and 100 ug/ml). In contrast, a randomized peptide (#5R) at the same concentration did not display any synergy with vancomycin.

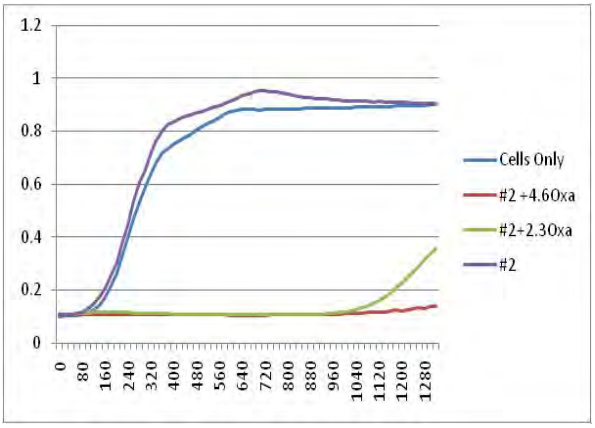

Figure 8. Overnight growth curves of NRS-382 cells (oxacillin MIC>16 ug/ml) were tested with 50 ug/ml peptide #2 with sub-MIC levels of oxacillin.

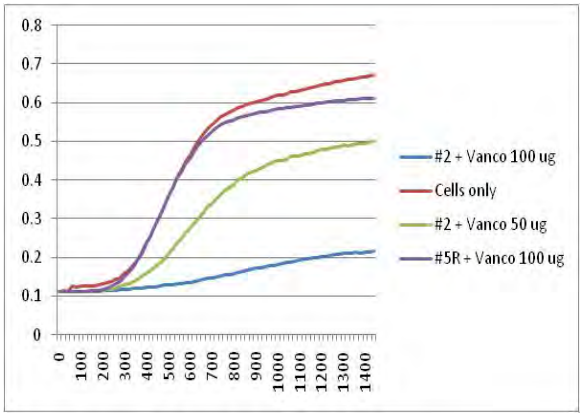

Figure 9. Overnight growth curves of VRS-1 cells (vancomycin MIC>256 ug/ml) were tested with 50 ug/ml peptide #2 with sub-MIC levels of vancomycin.

Task 3. Determine minimal binding requirements (months 11-15)

Despite early evidence that all 10 peptides showed promise (Fig. 1), and later evidence that suggested #5 and #39 were the best peptides (Figs. 2, 3, and 7), only peptide #2 displayed positive results in the Elisa assay (Fig. 1), binding to the staphylococcal surface (data not shown), and in synergy experiments with antibiotics (Figs. 8 and 9). Therefore, we chose to focus all our attention on peptide #2.

Peptide #2 did not share any conserved sequences with the other top 10 peptides except for adjacent lysine and proline residues (Fig. 4). Therefore, we attempted to synthesisze a poly-KP peptides of 6 residues in length (i.e. KPKPKP). Unfortunately, this peptide was not soluble in any aqueous buffer we tested and the organic buffers were not consistent with an environment where we could test the peptide against live staphylococcal cells. Thus, we decided to use the whole peptide #2 for our conjugation experiments in Aim 3.

<u>Aim 3. Conjugate final candidate peptides to vancomycin and evaluate "re-sensitization" of VRSA to vancomycin.</u>

Task 1. Conjugate peptides to vancomycin or vancomycin derivatives. (months 16-17)

After consulting several organic chemists, it was determined the best approach to conjugate our peptide to vancomycin was via a 2 step process using thiol linkage of a cysteine residue to a

bromine covalently linked to vancomycin. First we synthesized N-terminal as well as C-terminal cysteine derivatives of peptide #2 (i.e. CSPKHDNHTMRLK AND SPKHDNHTMRLKC). Next, we reacted the amine group of the vancosamine moiety on vancomycin with bromoacetic acid N-hydroxysuccinimide ester, which is commercially available (Fig. 10). Vancosamine is an amino sugar on the "backside" of vancomycin (i.e., it does not participate in binding to the di-alanine motif on the peptidoglycan). This complex was purified by HPLC and then mixed with either of the cysteine derivatives of peptide #2 at pH 8.0 and re-purified by HPLC. Our yield was ~50% using this method.

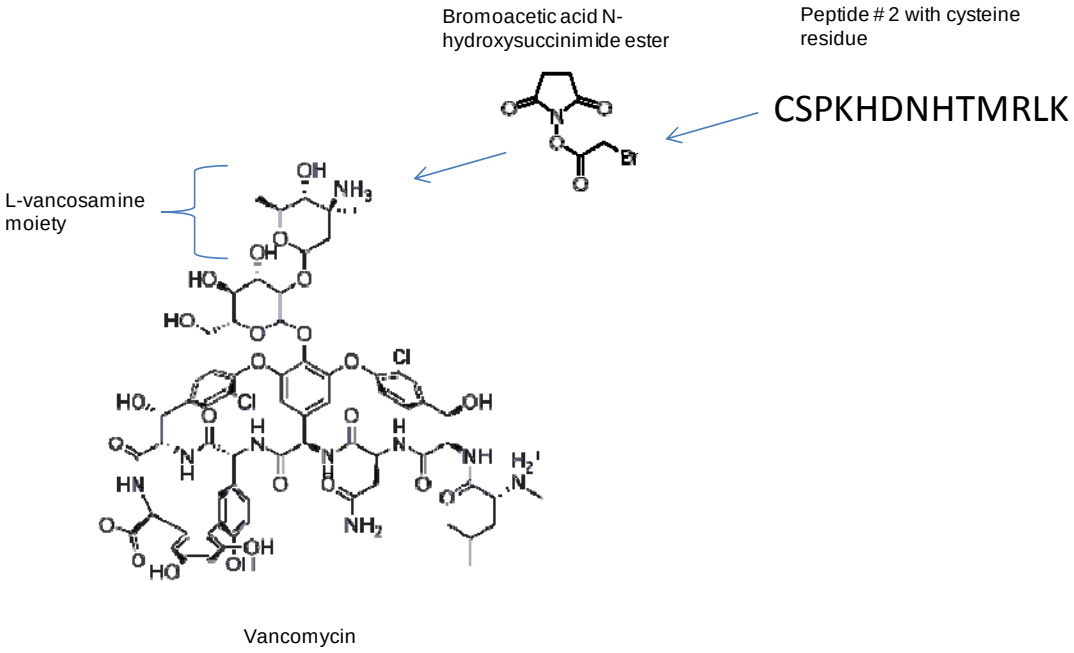

Figure 10. Strategy used to covalently link our peptide to vancomycin. First, the amine group of the L-vancosamine moiety was chemically reacted with bromoacetic acid N-hydroxysuccinimide ester to transfer the bromine group. In step two, our cysteine containing peptide binds the free bromine group.

Task 2. Test peptide/vancomycin complexes for sensitivity. (months 17-18)

In the final task, we treated VRSA strain VRS-1 with vancomycin as well as our vancomycin/peptide #2 complex. Unfortunately, we saw no difference between the two as both had overnight MIC values >256 ug/ml. However, we next tried a series of growth curve experiments. A representative example is shown in Fig. 11. At 256 ug/ml, both delayed growth of VRS-1, but did not completely inhibit growth at this concentration. However, it should be pointed out that vancomycin has a formula weight of 1450 whereas the vancomycin peptide complex has a formula weight of ~3000. Therefore, at identical concentrations, on a molar basis,

the complex only had half as much vancomycin yet had a similar effect on growth. Thus, there does appear to be some proof of principle evidence for the peptide/complex hypothesis. Note, only the complex with the N-terminal cysteine was formed as the one with the C-terminal cysteine proved to be insoluble in aqueous buffers.

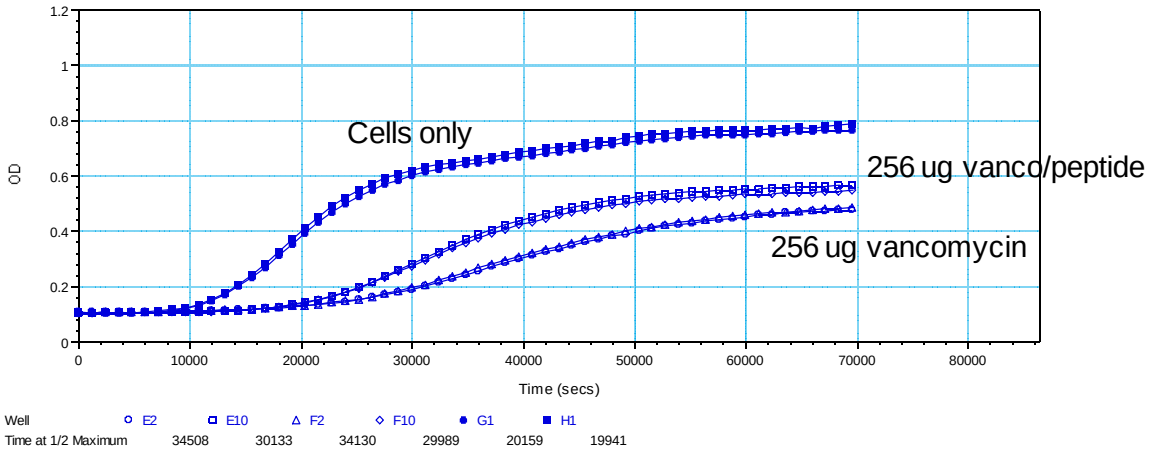

Figure 11. Overnight growth curves of VRS-1 exposed to 256 ug/ml vancomycin or 256 ug/ml vancomycin/peptide complex.

KEY RESEARCH ACCOMPLISHMENTS:

- Identified 40 peptides by phage display, which were later narrowed down to 10, that specifically bind pentaglycine preferentially over BSA controls
- Demonstrated binding of several synthesized peptides to the staphylococcal surface
- Demonstrated synergy of peptide #2 with oxacillin on MRSA and vancomycin on VRSA strains
- Made one soluble vancomycin/peptide #2 complex when a cysteine residue was added to the N-terminal side.
- Demonstrated similar profile of the complex compared to vancomycin on growth curves when identical concentrations were used.

REPORTABLE OUTCOMES:

Some aspects of our data and/or overall strategy have been presented at the following meetings/symposia during the past 18 months:

- Military Infectious Disease Research Program, Defense Health Program enhanced (DHPe), Wound Symposium, San Antonio, TX. Symposium speaker.
- Georgia Institute of Technology, Atlanta, GA. Invited speaker.
- The Rockefeller University, New York, NY. Invited speaker.
- Catholic University, Washington, D.C. Invited speaker
- U.S. Army Medical Research and Materiel Command/Military Infectious Diseases Research Program, Silver Spring, MD. Symposium speaker at the USAMRMC MIDRP W Wound Conference.
- Armed Forces Infectious Disease Society Annual Meeting, Annapolis, MD. Symposium speaker.
- National Institute of Standards and Technology, Gaithersburg, MD. Invited speaker.

In addition, we are submitting our results for peer review publication. Once the manuscript is accepted, I will forward a final copy to Dr. Dwayne Taliaferro to add to this file.

CONCLUSION:

Through bio-panning of the staphylococcal cell wall with a phage display library, we have identified 10 12-mer peptides that bind to the pentaglycine crossbridge of the staphylococcal peptidoglycan. While these peptides do not possess antibacterial properties when used alone, they do work in synergy with common antibiotics, thus allowing a lower therapeutic dose of antibiotic to be used, which in itself in an unexpected, but significant finding. We also showed several of our peptides can bind the staphylococcal surface, which was the goal of screening them against the pentaglycine mimetic in the first place. Finally, we conjugated one peptide to vancomycin via a thiol linkage and showed it retained similar properties to unconjugated vancomycin. While the addition of the peptide was not able to lower the MIC values, we are nonetheless encouraged because the complex is twice the mass of vancomycin, so at identical concentrations, the amount of vancomycin used in the complex was only half as much as needed for uncomplexed vancomycin. While the complex could not lower the amount of vancomycin needed to therapeutic levels, it does show proof-of-principle for the method and indicates that further research in this area is warranted.

<u>The way forward</u>

Although the project period has ended and all of the tasks were addressed, I do plan to press forward with this line of research. First and foremost, we plan expanding our synergy studies on both MRSA and VRSA strains with all of the peptides. The synergy finding was not envisioned as part of the original proposal, but after the peptides failed to display antimicrobial properties on their own, we decided to see if they had any synergy with antibiotics, which turned out to be a serendipitous finding which warrants additional investigation. Also, we can go back and conjugate peptides #5 and #39 to vancomycin now that we have worked out the methodology. These peptides showed initial promise, but we chose peptide #2 to advance for Aim 3. Perhaps

once conjugated to vancomycin, these peptides will prove better than peptide #2. Finally, we are interested in planning a new round of bio-panning with a phage display library. However, this time we would use a library that contains multiple cysteine residues which will constrain the peptides in a more rigid structure similar to how they are displayed on the surface of the phage.

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APPENDICES:

None.

SUPPORTING DATA:

Figures and figure legends are contained within the body of the text above.